Detection of *Illicium anisatum* as Adulterant of *Illicium verum*

Author

Natascha Techen[1], Zhiqiang Pan[2], Brian E. Scheffler[3], Ikhlas A. Khan[1]

Affiliation

[1] National Center for Natural Products Research and Research Institute of Pharmaceutical Sciences, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS, USA
[2] USDA-ARS-NPURU, University, MS, USA
[3] USDA-ARS-MSA, Stoneville, MS, USA

Correspondence

Dr. Natascha Techen
University of Mississippi
National Center for Natural Products Research
School of Pharmacy
P.O. Box 1848
University
MS 38677
USA
Tel.: +1-662-915-1549
Fax: +1-662-915-7062
ntechen@olemiss.edu

Abstract

▼

Chinese Star anise, *Illicium verum* Hook, is a well known spice in many cultures and has also been used to treat infant colic. Recent publications report that Chinese Star anise might be adulterated with the toxic Japanese Star anise, *Illicium anisatum* L. We have developed a molecular method that helps with the detection of *I. anisatum* as adulterant of *I. verum*. We PCR-amplified the internal transcribed spacer (ITS) region and analyzed it with the endonucleases *Pst*I and *Bfm*I. Based on fragment length polymorphisms (RFLP), we were able to detect and distinguish between *I. anisatum* and other *Illicium* species in powdered samples.

Introduction

▼

Roche Pharmaceuticals is the current sole manufacturer of Tamiflu®, a pharmaceutical known to lessen the severity of avian flu. The production of Tamiflu® is based on extraction of shikimic acid from Chinese star anise (*Illicium verum* Hook f.) and Japanese star anise (*Illicium anisatum* L., syn. *I. japonicum* Sieb, syn. *I. religiosum* Sieb et Zucc., syn. Shikimi fruit). Star Anise trees, and thus its crop, had been nearly completely destroyed by a series of severe tropical storms [1], [2]. Since demand for Tamiflu® is growing, there is an increased risk that batches of *I. verum*, either intentionally or unintentionally, are adulterated with *I. anisatum* fruits. In fact, the FDA recently advised that consumption of tea brewed from Chinese Star Anise carried risks because of reported cases of illness. However, the distinction between these two species of *Illicium* is not of concern for the purpose of extraction of shikimic acid but it is for food use.

The eight-pointed stellate fruit Chinese Star Anise (*I. verum*) has been used in the treatment of infant colic and is a well-known spice in many cultures. It is commonly known as being safe and non-toxic. In contrast to Chinese Star Anise, the closely related and similar in appearance species Japanese Star Anise (*I. anisatum*) is known to have both neurological and gastrointestinal toxicities [3] because of its sesquiterpene anisatin content. The possible adulteration of Chinese Star Anise with Japanese Star Anise is strongly supported [4].

It is very difficult to detect fragments of Japanese Star Anise fruit in powdered Chinese Star Anise fruit. Various methods have been published to identify and distinguish between them, such as morphological, chemical analysis by fluorescent microscopy, gas chromatography and an HPLC/ESI-MS/MS method [5], [6]. In addition to these methods, we have developed a molecular method to distinguish and identify Japanese Star Anise as an adulterant of Chinese Star Anise.

Material and Methods

▼

Plant material

The *Illicium* spp. samples used in this study were obtained from Trish Flaster Botanical Liaisons, Boulder CO (*I. anisatum* #2726 and *I. verum* #147; whole fruit, China); from Dr. M. Iinuma Gifu Pharmaceutical University, Japan (*I. anisatum* #1073; whole fruit, Japan); from Dr. Hao Gang, University of Hong Kong (*I. lanceolatum* Smith #3307; leaf, Wuhan Botanical garden, China), (*I. macranthum* Smith #3308; Xishuangbanna Botanical garden, China), (*I. arborescens* Hayata #3305; leaf, Taizhong, Taiwan); from Richard Saunders, University of Hong Kong (*I. dunnianum* Tutch. #3314; leaf, Chi-

na), (*I. leiophyllum* #3313; leaf, China) and collected by Dr. Natascha Techen University of Mississippi (*I. floridanum* Ellis # 3839; leaf, Mississippi). At a local Asian grocery, dried star anis seed was purchased from four different manufacturers (Hop Lee Trading Co., Inc NY #3309; Sincere Trading LLC, NY #3310; Ho's Trading Inc. NY #3311; Mass Dragon Trading Company, China #3312). Voucher samples of the *Illicium* spp. are deposited at the National Center for Natural Products (NCNPR), University of Mississippi.

DNA extraction and PCR conditions

Whole fruits or leaves of *Illicium* spp. were ground to a fine powder with two stainless steel metal balls for 1 min at amplitude 100 with an MM 2000 (Retsch, Germany). DNA was extracted with a modified CTAB method [7], [8]. Ten ng of DNA were used as template for PCR. PCR reactions were performed in 25 μL reactions [9]. After amplification, each PCR reaction was analyzed by electrophoresis on a 1 – 3% TAE agarose gel or on a 4 – 20% TBE polyacrylamide "PAGE" Criterion Precast Gel (Biorad cat. no.: 345 – 0061) and visualized under UV light. The sizes of the PCR products were compared to the molecular size standard 1 kb plus DNA ladder (cat no.: 10787 – 018, Invitrogen).

The nuclear internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS) were amplified from genomic DNA using the forward primer Ili-ITS-F0 (TCTTGGAGGTGATGGGAATG) and reverse primer Ili-ITS-R1 (AGAGTTCTTGTGCCACTAGC). PCR reactions were purified, extracted and cloned as described in [9]. From each transformation event, DNA from three to eight single colonies was isolated with the Spin Miniprep Kit (cat no.: 27106, Qiagen Inc.) and double stranded sequenced on an automated DNA sequencer (model ABI 3730XL; Applied Biosystems), and at GENEWIZ, Inc. Restriction digestions were performed following the manufacturer's recommendations in 40 μL reactions with 15 μL PCR product (300 – 400 ng) and one unit of enzyme. Twenty μL of the reaction mixture were either analyzed by agarose gel electrophoresis or PAGE.

Sequence analysis

Sequences were derived from Gene Bank: *Illicium* spp. (AF163729, AF163724, AF163723, AF163728, AF163732, AF163725, AF163726, AF163733, AF163727, AF163722), *Kadsura* spp. (AF263446, AF263448) and *Schisandra* spp. (AF263435, AF263440). For sequence analysis and alignment, the programs Seqman and MegAlign (DNASTAR 3.5 Inc.), GeneDoc version 2.6.002 [10] and ClustalW [11] were used. Homology searches were performed with the program BLASTn [12].

Results

▼

To determine if areas in the ITS region could be used to design *Illicium* specific primers, alignments were performed with the already published ITS sequences from the genus *Illicium* spp. and the ITS sequences of their closest relatives *Schisandra* spp. and *Kadsura* spp. Based on the differences between the sequences, for *Illicium* spp. one forward Illi-ITS-F0 and one reverse primer Illi-ITS-R1 were designed. The primers were tested on DNA of eight different *Illicium* species: *I. anisatum*, *I. verum*, *I. lanceolatum*, *I. arborescens*, *I. macranthum*, *I. dunnianum*, *I. leiophyllum* and *I. floridanum*. From all of the tested samples, a PCR product with a size of > 0.6 kb was amplified. To verify the ITS region of *I. verum* and *I. anisatum*, all the generated PCR products were cloned and sequenced. The total length of the amplified PCR product from the *I. verum* samples was 570 bp. The PCR product from the *I. anisatum* samples had a length of 569 bp. The alignment between the ITS sequence of *I. anisatum* (AF163729), *I. verum* (AF163724) and the sequenced clones of *Illicium* spp. samples showed differences such as deletions, additions and nucleotide changes (○ **Fig. 1**).

To validate our *I. verum* sequence compared to the Gene Bank, the ITS region of four commercially available *I. verum* samples was analyzed. Over one hundred clones in total were sequenced. The results showed that these sequences were concordant with the previous sequencing results of *I. verum* (data not shown). Furthermore, the entire ITS region was amplified using the primers "ITS5" and "ITS4" as reported in Hao et al. [13] from *I. verum* and *I. anisatum* samples, and digested either with endonuclease *Hind*III or *Hinp*1I to analyze the restriction fragment pattern. Results showed that the detected fragment pattern did not match the predicted fragment pattern based on the published

Fig. 1 Sequence alignment between sequences derived from Gene Bank (AF163729 and AF163724) and the sequenced clones of *Illicium anisatum* and *Illicium verum*.

sequences AF163729 and AF163724 (data not shown). However, further analysis showed that the *I. anisatum* sequence AF163729 is a 100% match to the *I. fargesii* sequence AF163730 [13] indicating a possible mislabeling of the earlier Genbank submission. The alignment (○ **Fig. 1**) between the sequenced clones of *I. verum* and the sequenced clones of *I. anisatum* showed 99% homology. Differences were four nucleotide changes and a deleted nucleotide in the *I. anisatum* sequence. We investigated whether both the *Pst*I and *Bfm*I endonucleases generate restriction fragment length polymorphisms that can be helpful in distinguishing between *I. anisatum*, *I. verum* and other *Illicium* species. DNA from *I. anisatum*, *I. verum*, *I. lanceolatum*, *I. arborescens*, *I. macranthum*, *I. dunnianum*, *I. leiophyllum* and *I. floridanum* served as templates in a 35 cycle PCR amplification using the designed primers Illi-ITS-F0 and Illi-ITS-R1. PCR products of about 0.6 kb in length were detected from all samples and digested either with *Pst*I or with *Bfm*I. The analyses with the two endonucleases showed restriction fragment length polymorphisms (○ **Fig. 2**). As expected, all amplified PCR products generated from the various species were cut with *Pst*I into two fragments of about 175 bp and 395 bp in length. Only the *I. anisatum* PCR product appeared uncut (○ **Fig. 2A**). Also, all amplified PCR products generated from the various species were cut with *Bfm*I into the correct number of fragments as predicted. The *I. anisatum* PCR product was cut into two fragments of about 188 bp and 381 bp in length (○ **Fig. 2B**) and the PCR products for all other tested species produced three fragments of about 13 bp (not detectable), 175 bp and 381 bp in length. These results show that the use of the restriction endonucleases *Pst*I and *Bfm*I can be helpful to distinguish *I. anisatum* from the other analyzed *Illicium* species.

We investigated what amount of *I. anisatum* tissue could be detected as an adulterant of *Illicium verum* tissue. We extracted DNA from mixtures consisting of *I. verum*: *I. anisatum* with ratios from 1:1 to 1,000:1. This DNA served as template in a 35 – 45 cycle PCR amplification using the designed primers Illi-ITS-F0 and Illi-ITS-R1. PCR products were detected from all prepared mixtures and digested either with *Pst*I or *Bfm*I. Depending on which sample of *I. anisatum* (either sample 2726 or sample 1073) was mixed with the *I. verum* sample 147, the sensitivity of detection varied for the characteristic *Pst*I fragments of *I. anisatum*. For example, from mixtures of *I. verum* sample 147 with *I. anisatum* sample 2726, the characteristic 0.6 kb fragment was detected from digested PCR products of ratios of 1:1 to 500:1 (○ **Fig. 3A**). The detection limit was different when mixtures of *I. verum* sample 147 and *I. anisatum* sample 1073 were analyzed. For example, the characteristic 0.6 kb *Pst*I fragment from *I. anisatum* was detected from digested PCR products of ratios of 1:1 to 50:1 (○ **Fig. 3B**). From mixtures of *I. verum* sample 147 with *I. anisatum* sample 2726, the characteristic 188 bp *Bfm*I fragment from *I. anisatum* was detected from digested PCR products of ratios of 1:1 to 500:1 (○ **Fig 4A**). The characteristic fragment from *I. anisatum* sample 1073 was detected from digested PCR products of ratios of 1:1 to 50:1 (○ **Fig. 4B**). The results are concordant with our *Pst*I digestion results. The experiments were repeated three times and the results verified.
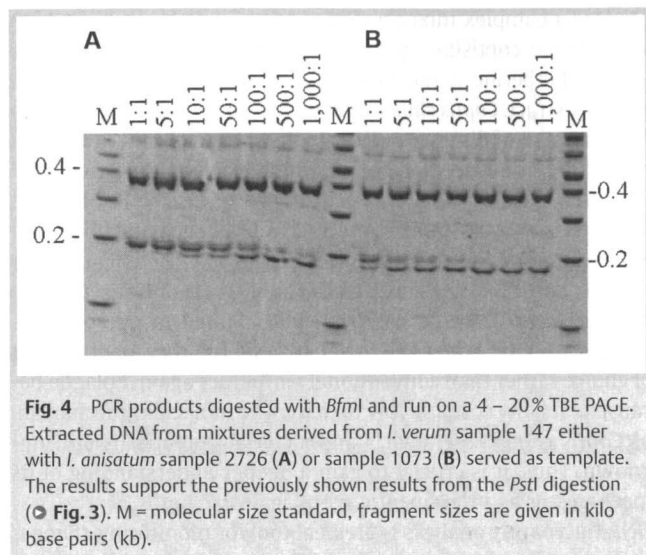
Discussion
▼

The purpose of the study was to determine if the ITS region of *I. verum* and *Illicium anisatum* can be used as a molecular marker for rapid identification. The ITS sequence from *I. anisatum* and *I. verum* was amplified by PCR and analyzed. We found that there was less than 100% homology to the sequences published in Gene Bank. Hao et al. [13] did not report the number of times the direct PCR sequencing was repeated to verify their sequencing results, however it is common in many polygenetic studies to
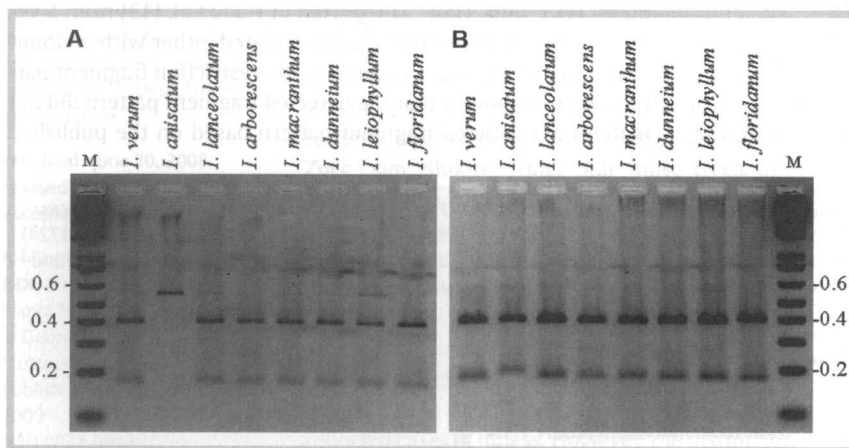
Fig. 2 Agarose gel image of *Pst*I (**A**) and *Bfm*I (**B**) digested PCR products. Single PCR products of ~600 bp were obtained when using various *Illicium* spp. DNA as template. With one exception, *I. anisatum*, all amplified PCR products were cut with *Pst*I into fragments of about 0.2 and 0.4 kb. M = molecular size standard, fragment sizes are given in kilo base pairs (kb).
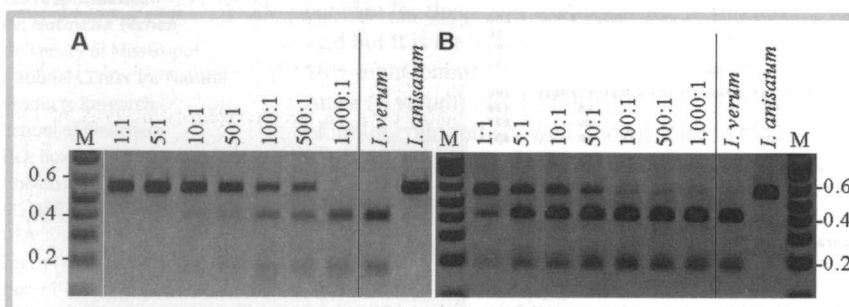
Fig. 3 Agarose gel image of *Pst*I digested PCR products derived from mixtures of *I. verum*: *I. anisatum*. From mixtures with *I. anisatum* sample 2726 (**A**) the detection limit was at 500:1. From mixtures with *I. anisatum* sample 1073 (**B**) the detection limit was at 100:1. The results from digestion with *Pst*I varied depending on which sample of *I. anisatum* 2726 or 1073 was mixed with the *I. verum* sample 147. M = molecular size standard, fragment sizes are given in kilo base pairs (kb).

Fig. 4 PCR products digested with *Bfm*I and run on a 4 – 20 % TBE PAGE. Extracted DNA from mixtures derived from *I. verum* sample 147 either with *I. anisatum* sample 2726 (**A**) or sample 1073 (**B**) served as template. The results support the previously shown results from the *Pst*I digestion (○ **Fig. 3**). M = molecular size standard, fragment sizes are given in kilo base pairs (kb).

analyze only one sample. In contrast, the *I. anisatum* sequence presented in this paper was verified from several independent plant samples using multiple cloning and sequencing events and for that reason it can be considered as the correct *I. anisatum* sequence. The authors suspect that the *I. anisatum* sequence published in Hao et al. [13] might represent a contamination derived from the *I. fargesii* sample.

Based on a five nucleotide difference, a restriction fragment length polymorphism (RFLP) helped us to distinguish *I. anisatum* from *I. verum*. This RFLP is also helpful to clearly distinguish *I. anisatum* from the other analyzed *Illicium* species. With the presented molecular technique, trace amounts of *I. anisatum* tissue, i.e., a ratio of 500 : 1, can be detected as adulterant of *I. verum*. Our results indicate that the detection limit can be variable depending on which samples of *I. anisatum* and *I. verum* are used to prepare the mixtures. This is not unexpected as it has been previously documented with other medicinal plants that the ability to detect trace amounts of a given species can be adversely affected by how the plant material is harvested, stored and processed [14]. Unfortunately we did not have the collection dates of some of the *Illicium* spp. samples, which would help us to determine the age of the sample and the length of the storage. We presented two options to generate RFLPs, either with *Pst*I or with *Bfm*I. To cut the amplified PCR product the use of the endonuclease *Pst*I (New England Biolabs, 10,000 units about $60) had the advantage that it is less expensive than *Bfm*I (Fermentas, 200 units about $60). In addition, the analysis can be made with an agarose gel because the generated *Pst*I PCR fragments from *I. verum* (0.2 kb and 0.4 kb) can be easily detected and distinguished from the uncut 0.6 kb *I. anisatum* PCR fragment. On the other hand, there is a clear disadvantage of using *Pst*I. If the Pst I restriction digestion of the *Illicium* PCR products is incomplete or fails, it results in a background of a 0.6 kb fragment that could be misidentified as *I. anisatum* PCR product. This could happen if the enzyme is degraded or the PCR product contains components that can hinder the activity of the restriction endonuclease. In the latter case, an after-PCR clean-up reaction might be beneficial. Such an inhibitor could come directly from the sample [14]. The use of *Bfm*I as the enzyme of choice to identify the adulteration of *I. verum* with *I. anisatum* has the advantage

that the PCR products from both species will be cut. If for any reason the enzyme *Bfm*I did not work appropriately it would be easily detected as un-cut PCR products. To be able to detect the 14 bp difference in the fragment length, a good separation method such as PAGE is required. The most appropriate analysis method depends on the user's budget and diagnostic needs.

Our primary goal for this study was to develop a molecular marker to distinguish the toxic Japanese star anise from the Chinese star anise. Our results showed that with the primer combination used, *I. anisatum* DNA can be detected as an adulterant of *I. verum* although the adulteration quantity cannot be precisely determined. To develop molecular marker(s) that could be used to differentiate *I. verum* from other *Illicium* species, different molecular biology strategies/techniques, such as AFLP or RAPD may be employed.

References
1 *Goodman PS.* Washington Post Foreign Service 2005: November 18: D01
2 *Zwitter I.* Bird flu drug Tamiflu's primary ingredient washed out in mudslides. Avant News Atlanta. Available online at http://www.avantnews.com/modules/news/print.php?storyid = 143; Accessed April 22, 2006
3 *Biessels GJ, Vermeij FH, Leijten FS.* Epileptic seizure after a cup of tea: intoxication with Japanese star anise. Ned Tijdschr Geneeskd 2002; 146: 808 – 11
4 *Ize-Ludlow D, Ragone S, Bruck IS, Bernstein JN, Duchowny M, Pena BM.* Neurotoxicities in infants seen with the consumption of star anise tea. Pediatrics 2004; 114: e653 – 6
5 *Joshi VC, Srinivas PV, Khan IA.* Rapid and easy identification of *Illicium verum* Hook. f. and its adulterant *Illicium anisatum* Linn. by fluorescent microscopy and gas chromatography. J AOAC Int 2005; 88: 703 – 6
6 *Lederer I, Schulzki G, Gross J, Steffen JP.* Combination of TLC and HPLC-MS/MS methods. Approach to a rational quality control of Chinese star anise. J Agric Food Chem 2006; 54: 1970 – 4
7 *Doyle JJ, Doyle JL.* A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 1987; 19: 11 – 5
8 *Cullings KW.* Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. Mol Ecol 1992; 1: 233 – 40
9 *Techen N, Khan IA, Pan Z, Scheffler BE.* The use of polymerase chain reaction (PCR) for the identification of *Ephedra* DNA in dietary supplements. Planta Med 2006; 72: 241 – 7
10 *Nicholas KB, Nicholas HB Jr, Deerfield DW II.* GeneDoc: Analysis and visualization of genetic variation. Embnet.news 1997; 4: 1 – 4
11 *Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG, Gibson TJ.* CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22: 4673 – 80
12 *Altschul SF, Gish W, Myers EW, Lipman DJ.* Basic local alignment search tool. J Mol Biol 1990; 215: 403 – 10
13 *Hao G, Saunders RMK, Chye ML.* A phylogenetic analysis of the *Illiciaceae* based on sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA. Plant Syst Evol 2000; 223: 81 – 90
14 *Techen N, Crockett SL, Khan IA, Scheffler BE.* Authentication of medicinal plants using molecular biology techniques to compliment conventional methods. Curr Med Chem 2004; 11: 1391